

AD _____

Award Number: W81XWH-16-2-0002

TITLE: Central Mechanisms and Treatment of Blast-Induced Auditory and Vestibular Injuries

PRINCIPAL INVESTIGATOR: Dr. Joseph B. Long

CONTRACTING ORGANIZATION: The Geneva Foundation
Tacoma, WA 98402

REPORT DATE: January 2017

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution is unlimited.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

| REPORT DOCUMENTATION PAGE | | | | Form Approved OMB No. 0704-0188 | |
|--|------------------|--------------------------|----------------------------|---|---|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS. | | | | | |
| 1. REPORT DATE January 2017 | | 2. REPORT TYPE Annual | | 3. DATES COVERED 30 Dec 2015 - 29 Dec 2016 | |
| 4. TITLE AND SUBTITLE Central Mechanisms and Treatment of Blast-Induced Auditory and Vestibular Injuries | | | | 5a. CONTRACT NUMBER | |
| | | | | 5b. GRANT NUMBER W81XWH-16-2-0002 | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) Dr. Joseph B. Long E-Mail: Joseph.b.long.civ@mail.mil | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES) The Geneva Foundation 917 Pacific Ave., Suite 600 Tacoma, WA 98402 | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited. | | | | | |
| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT As a consequence of advances in military medical care there are greatly increased numbers of survivors of blast-induced traumatic brain injury (bTBI) sustaining persistent neurosensory dysfunction including hearing loss and balance disorder. The study is to utilize our well-defined shock tube simulation of mild blast-induced traumatic brain injury (bTBI) in rodents to characterize interrelated biomechanical and pathophysiological mechanisms of blast-induced central auditory processing disorders (CAPDs) and central vestibular injuries (CVIs) and to develop an early therapeutic intervention for hearing loss and balance disorder mitigation. The major objectives of the proposed studies and relevant research sub-gaps are: 1) Verify the time course of hearing loss and balance disorders induced by blast exposure and define plasma and CSF TDP-43 as a biomarker related to blast-induced central auditory/vestibular deficits; 2) Characterize blast induced biochemical, functional and morphological alterations in central auditory/vestibular systems and establish that blast-induced altered expression of TDP-43 and its BDPs in these structures play a key pathophysiological mechanism leading to secondary injuries. | | | | | |
| 15. SUBJECT TERMS traumatic brain injury | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
| a. REPORT U | b. ABSTRACT U | c. THIS PAGE U | | | USAMRMC |
| | | | UU | | 19b. TELEPHONE NUMBER (include area code) |

Table of Contents

| | <u>Page</u> |
|--|-------------|
| 1. Introduction..... | 4 |
| 2. Keywords..... | 4 |
| 3. Accomplishments..... | 4-10 |
| 4. Impact..... | 10-11 |
| 5. Changes/Problems..... | 11 |
| 6. Products..... | 11 |
| 7. Participants & Other Collaborating Organizations..... | 12 |
| 8. Special Reporting Requirements..... | 12 |
| 9. Appendices..... | 12 |

1. Introduction

With widespread use of improvised explosive devices in recent military conflicts, blast-induced traumatic brain injury (bTBI) and neurosensory dysfunction have emerged as key military medical issues. Auditory and vestibular disorders are particularly prevalent, and the debilitating consequences of these injuries likely progresses with age. A comprehensive understanding of the structural and molecular components of the injury is essential for the development of the most appropriate therapies for auditory and vestibular deficits resulting from blast exposure. Existing data indicate that both the inner ear and the structures in the brain responsible for auditory and vestibular function are at high risk of injury following blast exposure. The proposed study will utilize an Advanced Blast Simulator (ABS) to recreate these injuries in rodents in the laboratory. Through comprehensive assessments of the resultant auditory and vestibular deficits using a battery of functional tests in conjunction with characterizations of the underlying biochemical and anatomical changes in these structures, the interrelated biomechanical and pathophysiological mechanisms responsible for blast-induced central auditory processing disorders (CAPDs) and central vestibular injuries (CVIs) will be elucidated and will provide therapeutic targets for hearing loss and balance disorder mitigation.

2. Keywords

blast overpressure, mild traumatic brain injury, auditory brainstem response, balance disorder, mouse, pathology, auditory cortex, brainstem, cerebellum, neuron

3. Accomplishments

○ What were the major goals for the project?

The major objectives for the project were: 1) to verify the time course of central auditory processing disorders (CAPDs) and vestibular injuries (CVIs) induced by blast exposure; define plasma and CSF TDP-43 as a biomarker related to blast-induced central auditory/vestibular deficits; 2) to characterize blast injury to primary auditory cortex and brainstem/cerebellum associated with CAPDs and CVIs; define blast-induced altered expression of TDP-43 as a key pathophysiological mechanism leading to the secondary central auditory and vestibular processing injuries.

Milestones:

Year 1: Obtain IACUC and ACURO approval of animal use protocol, define time-course of blast-induced auditory function deficits; define the role of TDP-43 in neuronal development.

Year 2: Assess time-course of vestibular functional disruptions, determine TDP-43 levels in serum and CSF, examine morphological alterations in specific neurons in AU, identify blast impaired functional connection between MGN and AU; examine the regulation of TDP-43 target genes.

Year 3: Examine morphological alterations of Purkinje neurons in the cerebellum; demonstrate blast impairments of functional connections between FL and Lat.

○ **What was accomplished under these goals?**

Bulleted list of key research accomplishments emanating from this research

- During this initial reporting period, we received approval of two animal use protocols, since all experimental objectives of the project are animal-based. The protocol 16-PN-20S, “Central mechanism and treatment of blast-induced auditory and vestibular injuries in mice” has been approved by WRAIR/NMRC IACUC and also has been accepted by ACURO. The protocol LIE-012-2016, “Molecular mechanism of synapse development and plasticity in animal models for traumatic brain injury” was approved by SoBran ACUC.
- We trained two research technicians who are now skilled in the experimental procedures required for this project, including neurobehavioral testing, animal euthanasia, pathological assessment and primary neuronal culture.
- We have assessed the impairments resulting from exposures to three closely-coupled blasts (19 psi) alone and in combination with weight drop (80 g, 1 m) using Auditory Brainstem Response (ABR) and Distortion Product Otoacoustic Emissions (DPOAE) in two strains of mice, CBA and C57BL.
- In this reporting period, we have begun the pathological evaluation of the effects of blast on the auditory cortex, brainstem and cerebellum (the principal brain regions associate with sound processing).
- We acquired preliminary data for characterization of blast-induced morphological alterations in cortical excitatory pyramidal neurons using Thy1-YFP transgenic mice that were provided by The Jackson Laboratory (JAX) and The Lieber Institution for Brain Development (LIBD).
- We also researched cellular mechanism(s) of neurondegeneration relevant to blast injuries. An *in vitro* experiment in which p21 CRISPR/dCas9 Lentiviral activation particles were transfected to the cultured primary cortical neurons. The preliminary data showed a possible role that p21 plays in cytoskeletal dynamics and Tau phosphorylation.
- To define the role of TDP43 in neuronal degeneration, a plasmid pLenti.CAG.EGFP-P2A-TDP43 which contains a neuron specific promotor CAG has been designed and generated by the collaborator at LIBD.
- The poster “Elevated expression of p21cip1 in blast neurotrauma might contribute to blast-induced Tauopathy” was presented at The Annual Symposium for Neurotrauma Society, Lexington, KY in June 2016.
- The poster “Characterization of auditory injury in mice exposed to blast overpressure in an advanced blast simulator” was presented at the annual Military Health System Research Symposium (MHSRS), Orlando, FL in August 2016.

Detailed experimental methods and results

➤ **Methods**

- **Blast-induced neurotrauma:** All animal experiments were conducted in accordance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals with an Institutional Animal Care and Use Committee approved protocol. The blast

overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) was generated by Valmex membrane rupture in the ABS which consists of a 0.5 ft long compression chamber that is separated from a 21 ft long transition/expansion test section (Fig. 1). For a single blast treatment (BOP), mice (male, 23 - 28 g) were secured in the ABS in a prone position facing the oncoming shockwave immediately after administration of 4% isoflurane gas anesthesia in an induction chamber for 8 min (O₂ flow rate 1.5L/min). For double blast exposures (BOP2), mice received additional isoflurane anesthesia for 2 min immediately following the first exposure and were then exposed to the second blast shockwave. For three blast exposures (BOP3), mice immediately received additional isoflurane anesthesia for 2 min separating the second and third blast exposures. Sham control animals were included in all individual experiments and were treated in the same fashion without exposure to blast shockwaves. For the combined blast and weight-drop injury (BW), the blast exposed mice (described above) was secured to the foam bed treated immediately afterwards by the 80 g cylindrical Plexiglas dropped from a 1 m height to the head

- **Auditory function assessment:** Auditory Brainstem Response (ABR) and Distortion Product Otoacoustic Emissions (DPOAE) testing was used to assess auditory function. Each mouse was tested under Ketamine/Dexdomitor anesthesia. Baseline ABR and DPOAE were recorded at 3 - 5 days before blast treatment. A time-course of blast effects on auditory function was assessed at 2, 7, 14 and 28 days after blast exposure.
- **Pathology:** Brains of mice was dissected after euthanasia and fixed in 4%PFA solution at the designed days post-injury. Coronal brain sections (40 - 100 μ m) were prepared using a vibrating microtome (Leica VT-1000S). Brain sections were processed for silver staining and immunohistochemistry.
- **Primary neuronal culture:** Female mice with timed 18 day pregnancies (E18) were anesthetized with 4% isoflurane in an induction chamber. The fetuses were decapitated immediate after removal from the uteri and placed in sterile Ca²⁺ and Mg²⁺-free Hanks balanced salt solution (HBSS). The cerebral cortex and hippocampus were isolated under a dissecting microscope. Cells were dissociated by incubation in HBSS containing 0.1% trypsin for 15 min followed by up and down pipetting in a 15 ml tube until no visible chunks of tissue remain. Cells were filtered, centrifuged and cultured in Neurobasal medium containing B27 serum free supplement.

➤ Results

• **ABS increase the righting reflex time in mice**

We investigated effect of ABS (Fig. 1) generated blast overpressure on righting reflex time (RRT) in mice (Fig. 2). The experimental groups were sham controls (Sham1, n = 30, Sham2, n = 8 and Sham3, n = 8), noise control (NC, n = 9), single blast exposure (BOP, n = 30), double blast exposures (BOP2, n = 10) and

triple blast exposures (BOP3, n = 30). Data showed that RRT increased significantly in blast treated groups, while there were no difference among sham controls as well as between sham and noise exposed mice. Compared to BOP, BOP2 and BOP3 caused a significant elevation of RRT ($p < 0.0001$), respectively. There was no significant change between BOP2 and BOP3. The mortality was 7.5% for BOP and 11.8% for BOP3.



Fig. 1. The Advanced Blast Simulator (ABS)

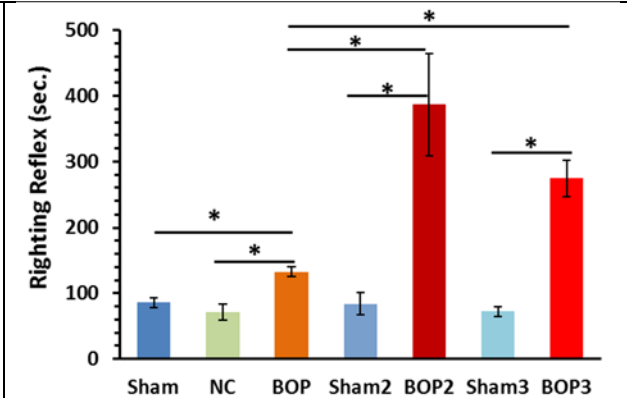


Fig. 2. Righting reflex time after exposure to ABS

- **Blast-induced elevation of ABR threshold**

Although C57BL mice have been reported to possibly have an age-related ABR change, they have nevertheless been recognized as a good strain for genetic and neurobehavioral research. CBA mice are commonly used in ear studies. During this reporting period, we have investigated the time-course of auditory functional deficits induced by blast overpressure in both strains of mice. Similar to the CBA mice (Fig. 3), C57BL mice presented significant ABR threshold shifts (Fig. 4) induced by blast exposure. A complete hearing loss (threshold > 90 dB) was observed at 2 days after three blast exposures and these deficits persisted over 3 months in the two strains of mice. Compared to the sham controls, blast-induced elevation of ABR threshold showed throughout the whole spectra of sound frequencies. At 14 days after injury, the ABR threshold to lower frequency (8 KHz) stimuli indicated a partially recovery of hearing. In contrast, the ABR threshold to higher frequency (40 KHz) stimuli did not recover through 90 days post-injury. The results indicate that the impact of blast exposure on high frequency (40 kHz) hearing was severe and persistent. DPOAE data also indicated that hearing loss occurred at 2 days after blast exposure and extended throughout 90 days post-injury (Fig. 5.)

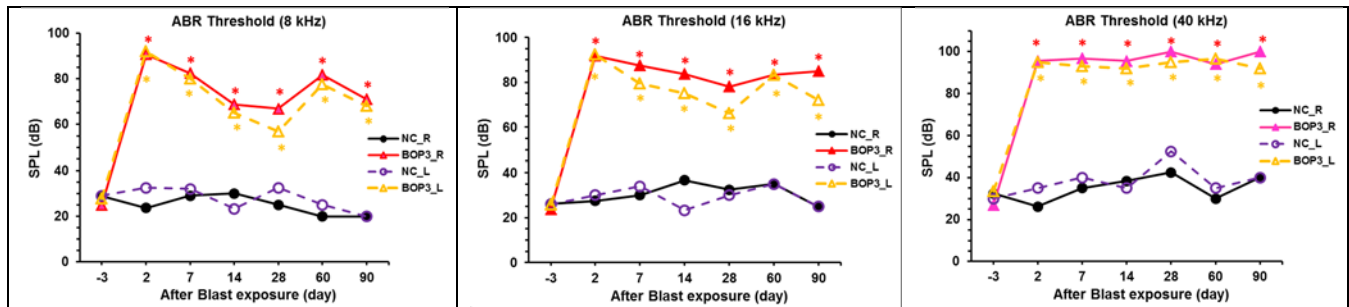


Fig. 3. Effects of three blast exposures on ABR threshold in CBA mice.

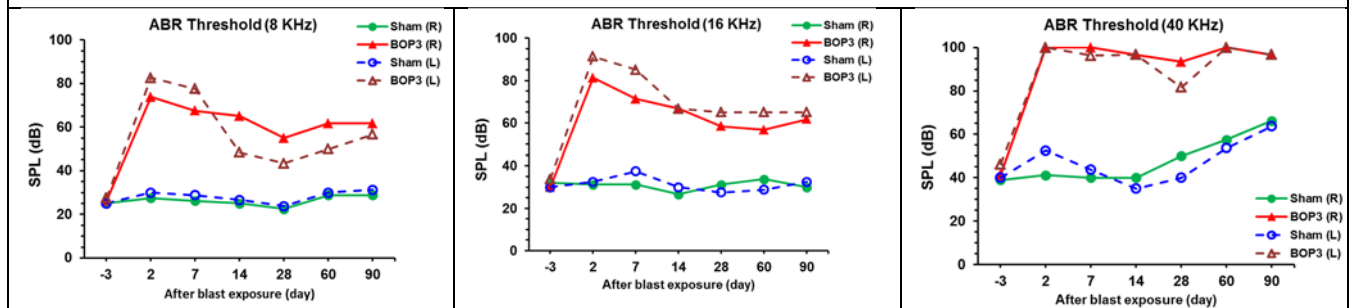


Fig. 4. Effects of three blast exposures on ABR threshold in C57BL/6 mice.

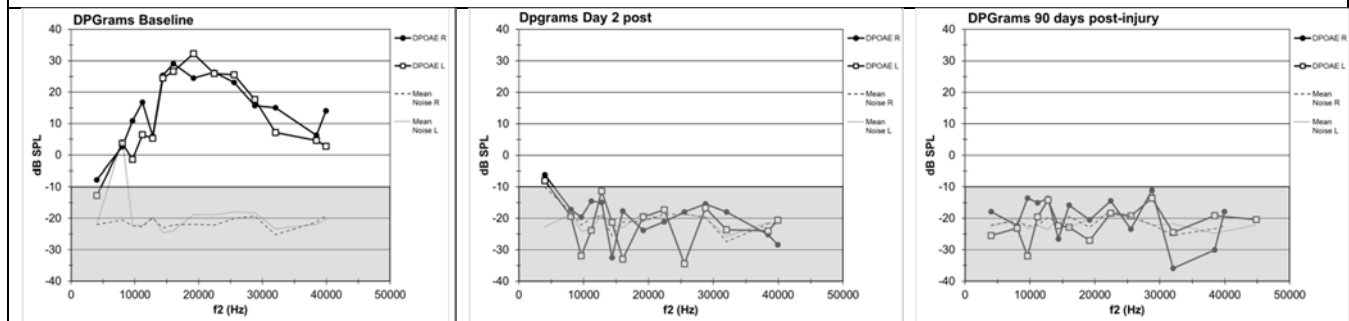


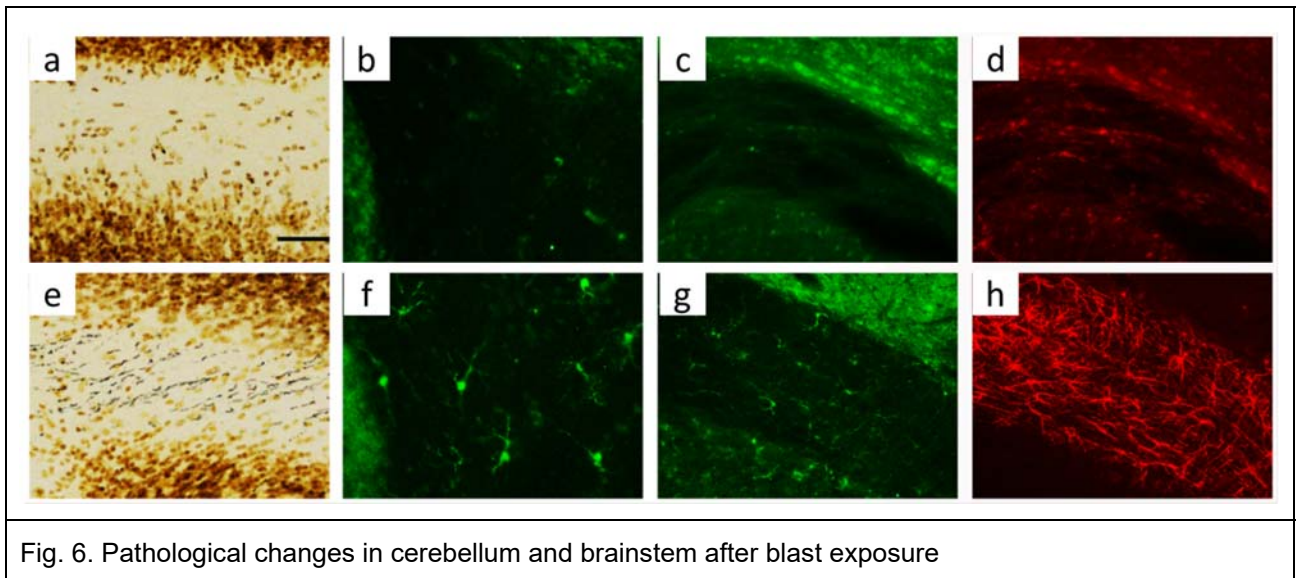
Fig. 5. Effects of three blast exposures on DPOAE in CBA mice.

• **ABS induces pathological changes in brainstem and cerebellum**

To evaluate blast damage to CNS, numerous antibodies have been purchased for evaluations of mouse brain sections. In this initial period, we have verified that antibodies GFAP (#3670, cell signaling), Iba1 (#01919741, Wako chemicals) and TDP43 (#PA116996, ThermoFisher) can be successfully used for immunohistochemistry (IHC) study in mouse. Antibodies targeting Tau (#4019, cell signaling), TDP43 (#ab42474, abcam) are currently excluded from the IHC study. Some antibodies such as PSD95, Tau-5, Neurexin 1 and p21 need to be further characterized for future applications.

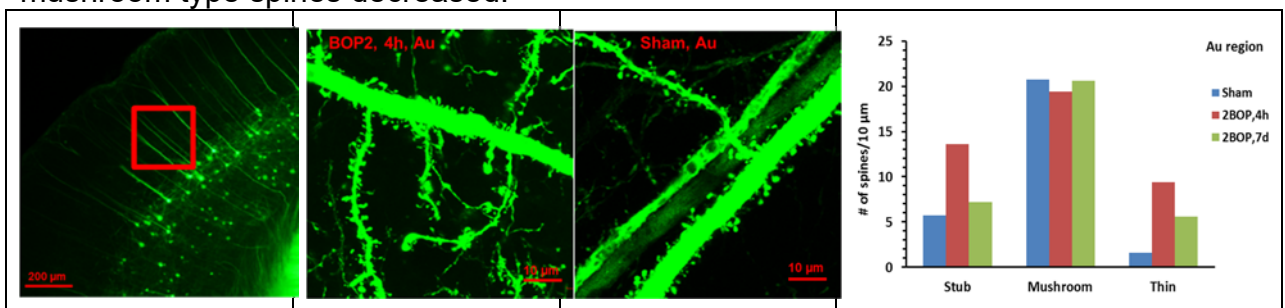
Our initial evaluation of brain sections at 7 and 14 days post-injury showed prominent axon degeneration in the white matter of cerebellum (which was detected by silver staining) and the proliferation of microglia and astrocytes in the cerebellum and brainstem regions (which were visualized by anti-Iba1 and anti-GFAP immunostaining, respectively). Those morphological changes were most pronounced in the experimental BOP3 group, but not in BOP group. Figure 6 illustrates the effect of blast shockwaves on axons and glial cells in cerebellum

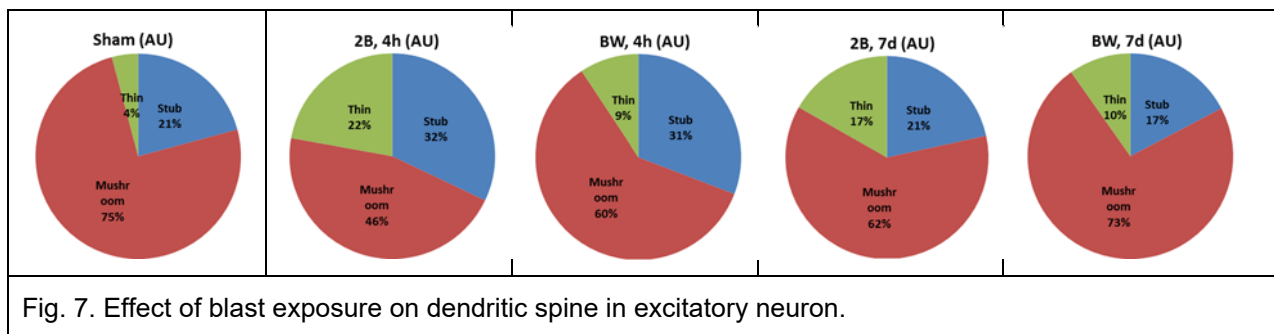
(a, b, e and f) and brainstem (c, d, g and h) at 14 days post-injury. Compared to sham control (a – d), blast exposure causes axonal degeneration (e) along with increased Iba1 (f, g) and GFAP (h) immunoreactivity, scale bar 100 μ m.



- **ABS induces the changes in excitatory neuron in auditory cortex**

Precise sensory information processing requires complex interactions between neurons. Dendritic spine numbers and shapes correlate with the strength of synaptic transmissions that are associated with the function of particular neural networks. Auditory cortex (Au) is one of the key units of sound processing. A Thy1-YFP mouse expresses yellow fluorescent protein at high levels in the layer V pyramidal neurons of the Au. During this reporting period, we acquired 16 transgenic mice with Thy1-YFP from the Jackson Laboratory and LIBD/JHU and examined blast-induced morphological alterations. The initial data (Fig. 7) showed that total number of dendritic spines in Au region was increased at 4h post-injury. Changes were post prominent in the immature types of stubby and thin spines, so that the ratio of mushroom type spines decreased.





• p21^{Cip1} might Contribute to Blast-induced Tauopathy

Although significant increases in tau and phosphorylated tau (p-Tau) proteins have been described in multiple brain regions following blast traumatic brain injury (bTBI) in rodents, the mechanism(s) underlying these changes is still undefined. Recent evidence suggests that an abnormal reactivation of the cell cycle may precede and cause the hyperphosphorylation and filament formation of tau protein in Alzheimer's disease and other tauopathies. To explore possible involvement of the proteins involved in cell-cycle progression in these mechanisms, we investigated the effect of p21^{Cip1}, a cyclin-dependent kinase inhibitor, on Tau expression. Our data showed that Tau and pTau were up-regulated significantly when p21 was overexpressed (Fig. 8), while Tau and pTau were down-regulated by knockout p21^{Cip1} in which p21 CRISPR/Cas9 was transfected to the cultured primary neurons (Fig. 9). These combined results indicate that p21^{Cip1} may play significant roles in cytoskeletal dynamics and apoptosis in addition to cell cycle regulation. In particular, TBI-induced overexpression of p21^{Cip1} might contribute to tauopathy.

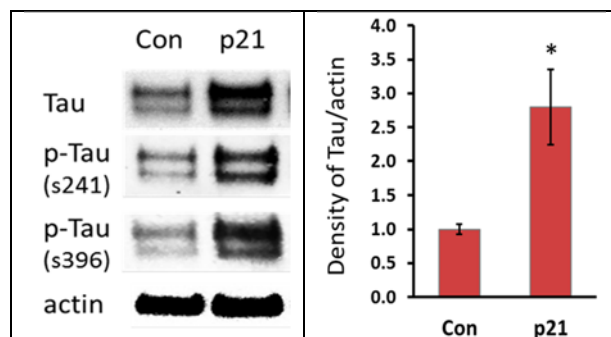


Fig. 8. Effect of overexpression of p21 on Tau and pTau expression

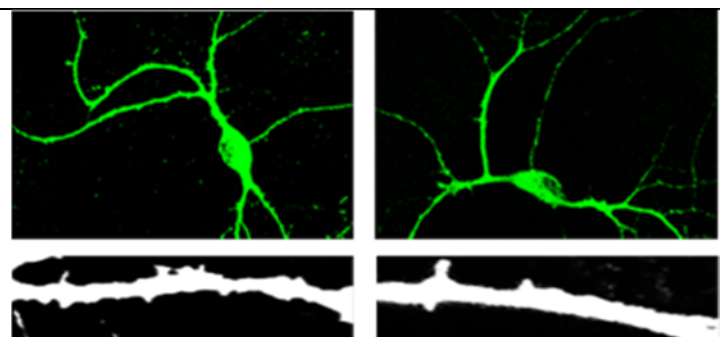


Fig. 9. Decrease in Tau expression and dendritic spines after knockout p21

4. Impact

To address fundamental questions about how blast-induced altered expression of TDP-43 may be a key pathophysiological mechanism leading to the secondary central auditory and vestibular processing injuries, we made pLenti-CAG-EGFP-P2A-TDP43 and pLenti-L7-EGFP-P2A-TDP43 constructs to transduce them to mouse primary cultured cortical neurons and primary cerebellar Purkinje neurons, respectively, to examine if overexpression of TDP-43 has an impaired effect on dendritic development. We also made pLenti-CAG-EGFP and pLenti-L7-EGFP constructs as empty control

plasmids to transduce them to mouse primary cultured neurons as mentioned above. Our preliminary data showed that both two control constructs resulted in very high GFP expression in cultured neurons. Surprisingly, we didn't see any GFP positive neurons in culture dishes after transduction of TDP-43 into cortical neurons and Purkinje neurons, respectively. Under the circumstances, TDP-43 expression was also difficult to be measured by Western blotting although several different TDP-43 antibodies were applied. We speculate that there are at least two possibilities leading to this unanticipated outcome: 1) overexpression of TDP-43 has a strong toxic effect leading to neuron dead; 2) co-expression of EGFP and TDP-43 doesn't work properly in the same plasmid due to an unknown mechanism, such as mutual interference leading to less GFP and TDP-43 expression in the same neurons. Now, we are working on new constructs containing TDP43 with small tag, pLenti-CAG-TDP43-Myc and pLenti-L7-TDP43-Myc constructs. We will co-transduce new constructs with the afore-mentioned empty control plasmids into primary cultured neurons to address if there is mutual interference in the same plasmid. If TDP-43 expression can still not be detected, we will be pretty sure that TDP43 has a strong toxic effect on neuronal survival and development. If so, we will reduce amount and duration to apply TDP-43 constructs for primary cultured neurons, and will examine the impaired effect of small amount of TDP-43 expression on neuronal dendritic development.

5. Changes/Problems

None to note

6. Products:

Lay Press

None

Peer-Reviewed Scientific Journals

None

Books or other non-periodical, one-time publications

None

Other publications, abstracts, conference papers and presentations

1. Y Wang, Y Su, Y Wei, D Wilder, I Gist, P Arun and J Long. Elevated expression of p21cip1 in blast neurotrauma might contribute to blast-induced Tauopathy. The Annual Symposium for Neurotrauma Society, Lexington, KY June 2016
2. Y Wang¹, Y Wei¹, S Van Albert¹, T Fitzgerald², A Northrop², R Urioste¹, P Arun¹, D Wilder¹, S Venkatasivasaisujith¹, I Gist¹, S McInturff², W Chang², M Kelley² and J Long¹. Characterization of auditory injury in mice exposed to blast overpressure in an advanced blast simulator. The Annual Military Health System Research Symposium (MHSRS), Orlando, FL. August 2016

3. 1 (Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, WRAIR, Silver Spring, MD)
4. 2 (Porter Neuroscience Research Center, NIDCD/NIH, Bethesda, MD)

Websites or other internet sites

Technologies or techniques

Inventions, patent applications, and/or licenses

7. Participants & Other Collaborating Organizations

| Name | Project Role | Percent Effort | Organization |
|-----------------|--------------------|----------------|--------------|
| Dr. Joseph Long | PI | 10% | WRAIR |
| Ying Wang | Co-PI | 30% | WRAIR |
| Yanling Wei | Research Associate | 50% | Geneva |
| Donna Wilder | Lab Manager | 50% | Geneva |
| | | | |

| Organization Name | Location | Contribution |
|--|----------|---------------|
| Lieber Institute for Brain Development at Johns Hopkins University | Maryland | Collaboration |
| | | |
| | | |

8. Special Reporting Requirements

A Quad Chart is attached.

9. APPENDICES

Two posters are attached.

Central mechanisms and treatment of blast-induced auditory and vestibular injuries

MR141274

W81XWH-16-2-0002

PI: Joseph B. Long

Org: WRAIR/The Geneva Foundation

Award Amount: \$1,476,364



Study/Product Aim(s)

The etiology of blast-induced hearing loss and balance disorders is largely undefined. There are no FDA-approved drugs for treatment. This study will utilize a well-characterized, high fidelity rodent blast injury model to evaluate central auditory processing disorders (CAPDs) and central vestibular injuries (CVIs) and target disrupted TDP-43 and PERK-eIF2 α -ATF4 signaling as a likely therapeutic means to mitigate blast-induced auditory and vestibular dysfunction.

Approach

Blast TBI model: repetitive blast overpressure exposures to mice

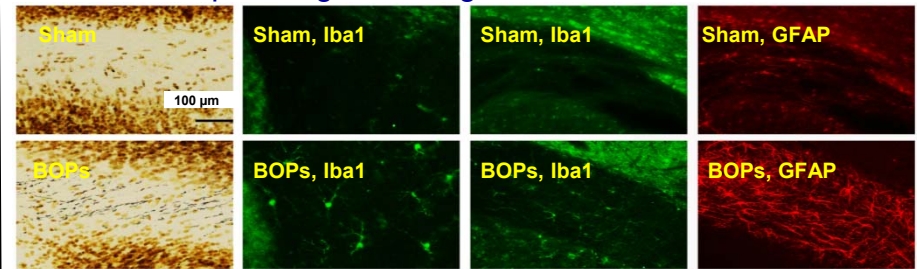
Functional assessment: ABR, DPOAE, VsEP and Rotarod

New technology: optogenetics with whole-cell patch recording to uncover the impaired functional connection between brain regions; CRISPR/Cas9 gene editing and Single cell RNA-seq assay

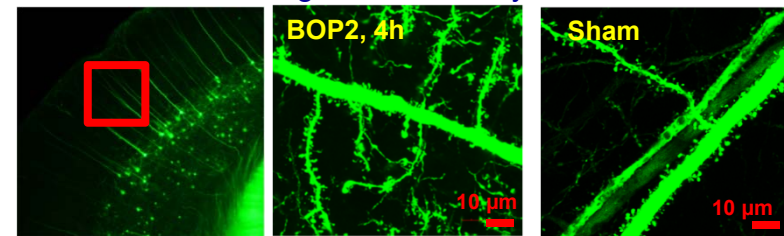
Pathology: silver staining, immunohistochemistry on transgenic mice for specific neuronal plasticity and morphology

Define biomarkers: Western blotting and ELISA

Blast-induced pathological changes in cerebellum and brainstem



Blast-induced changes in excitatory neurons in auditory cortex



Timeline and Cost

| Activities | CY | 16 | 17 | 18 |
|---|----|-------|-------|-------|
| Verify blast-induced CAPDs and CVIs, define TDP-43 levels in serum and CSF | | | | |
| Verify blast-induced morphological alterations in central auditory system and vestibular system | | | | |
| Estimated Budget (\$K) | | \$500 | \$500 | \$476 |

Updated: (01/27/2017)

Goals/Milestones

CY16 Goal

- ☒ Approval of animal use protocol
- ☒ Time-course of blast-induced auditory function deficits
- ☒ Define the role of TDP-43 in neuronal development

CY17 Goal

- ☐ Time-course of vestibular function assessment
- ☐ Determine TDP-43 levels in serum and CSF
- ☐ Morphological examination on specific neurons in AU
- ☐ Blast impaired functional connection between MGN and AU
- ☐ Examine the regulation of TDP-43 target genes

CY18 Goal

- ☐ Morphological examination on Purkinje neurons in the cerebellum
- ☐ Blast impaired functional connection between FL and Lat

Budget Expenditure to Date

Projected Expenditure: \$492,121.32

Actual Expenditure: \$410,722.93

Characterization of Auditory Injury in Mice Exposed to Blast Overpressure in an Advanced Blast Simulator

Ying Wang¹, Yanling Wei¹, Rodrigo Urioste¹, Stephen Van Albert¹, Amy Northrop², Sajja Venkatasivasaisujith¹, Yan Su¹, Donna Wilder¹, Peethambaran Arun¹, Irene Gist¹, Stephen McInturff², Weise Chang², Tracy Fitzgerald², Matthew Kelley² and Joseph Long¹

1. Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, WRAIR, Silver Spring, MD
2. Porter Neuroscience Research Center, National Institute on Deafness and Other Communication Disorders, Bethesda, MD

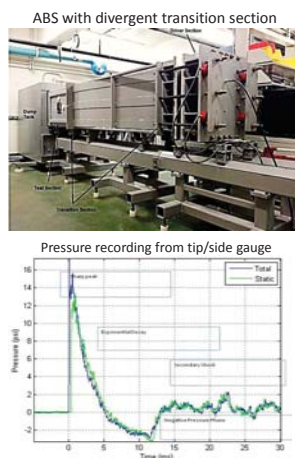


ABSTRACT

A high fidelity animal model is critical to define the mechanism(s) of blast-induced auditory injury and to develop therapeutic strategies. The present research is aimed at producing a comprehensive characterization of auditory functional deficits and associated pathological changes in the peripheral and central auditory signal processing regions disrupted by exposure to blast shockwaves. We have investigated the time-course of blast effects on auditory function and structural changes. Isoflurane anesthetized CBA mice (male, 8 weeks) were exposed to blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) generated by the Advanced Blast Simulator (ABS). Auditory function was assessed by analyzing distortion product otoacoustic emission (DPOAE) and auditory brainstem response (ABR) under anesthesia. Data showed that DPOAE signals were undetectable acutely after blast exposure and their disappearance persisted over 14 days that suggests the injuries to the inner ear. Blast exposure caused significant elevations of ABR threshold, increased ABR wave latency, and reductions in ABR wave amplitude immediately following the blast shockwave insult. These changes were observed over the entire acoustic frequency spectrum and persisted over 14 days. Immunostaining of Myo7a and Phalloidin in whole-mount cochlea revealed appreciable damage to hair cells, as well as to other structures in the inner ear. Increases in GFAP, Iba1 and axonal degeneration were detected in the brainstem and cerebellum at 14 days post-injury. The results indicate that both peripheral and central auditory signal processing regions are vulnerable to blast overpressure exposure in the ABS. This mouse model of blast-induced auditory injury should provide a useful experimental tool for studying the mechanisms underlying hearing impairment after blast exposure and for evaluating potential strategies for prevention and cure.

BACKGROUND

Nearly 60% of blast TBI victims exhibit hearing loss, tinnitus, dizziness and balance disorders. Despite the high incidence of auditory dysfunction resulting from blast injuries, the neurobiological mechanisms underlying these blast injuries are largely undefined. A high fidelity animal model is critical to define the mechanism(s) of blast-induced auditory injury and to develop therapeutic strategies. However, conventional shock tubes are limited in their ability to simulate explosive blast waveforms and flow conditions. The Advanced Blast Simulator (ABS) incorporates design features which allow higher fidelity replication of the key features of blast wave flow conditions, including the negative phase and secondary shock. ABS consists of a 0.5 ft long compression chamber that is separated from a 21 ft long transition/expansion test section by rupturable Valmex membranes.



METHODS

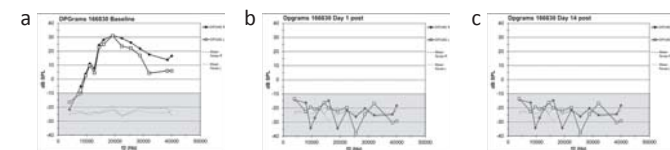
Animals and ABS: After anesthetization with isoflurane for 8 minutes, CBA mice (male, 23 - 28 g) were secured in the ABS in a prone position facing the oncoming shockwave. The blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) was generated by Valmex membrane rupture in the ABS. Sham controls were handled similarly but without exposure to the blast.

Auditory functional assessment: A time-course of blast effects on auditory function was assessed by analyzing auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) under Ketamine/Dexdomitor anesthesia.

Pathological investigation: At 14 days post-injury, the whole-mount cochlea were subjected to immunohistochemistry, and brain sections were evaluated after silver staining and immunohistochemistry.

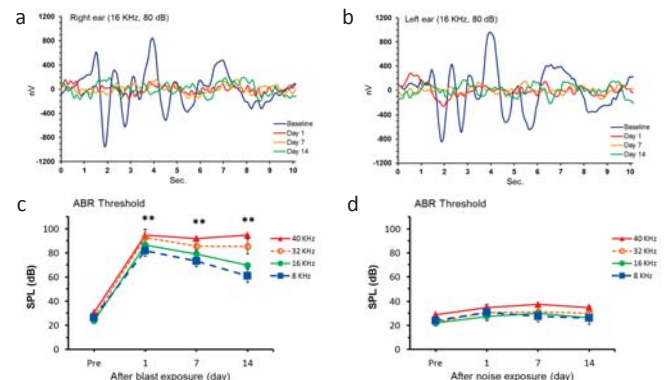
RESULTS

Figure 1. Blast exposure impaired DPOAE



Compared to its baseline (a), DPOAE was undetectable at 1 day (b) and 14 days (c) post-injury.

Figure 2. Blast exposure impaired ABR



RESULTS

Figure 3. Changes in cochlear hair cells after blast exposure

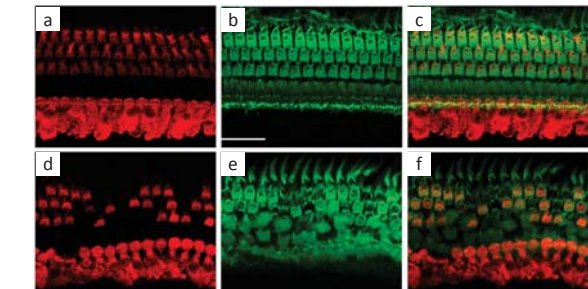
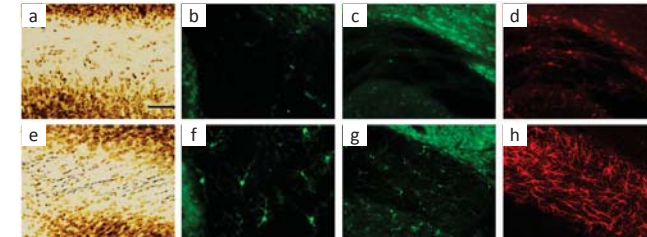


Figure 4. Changes in CNS after blast exposure



Effect of blast shockwave on axons and glial cells in cerebellum (a, b, e, f) and brainstem (c, d, g, h) at 14 days post-injury. Compared to sham control (a – d), blast exposure induces axonal degeneration (e), increases Iba1 (f, g) and GFAP (h), scale bar 100 μm.

CONCLUSIONS

- Blast shockwaves (19 psi) produced ABR threshold shifts that persist through 14 days. Compared to high frequency (40 kHz) hearing loss after blast exposure, low frequency (8 kHz) hearing recovered early.
- Appreciable damage to cochlear outer hair cells, inner hair cells, and other structures in the inner ear was observed. Blast overpressure generated by the ABS causes mild axonal degeneration and glial cells proliferation.
- This mouse model of blast-induced auditory injury should provide a useful experimental tool for studying the mechanism of hearing impairment after blast exposure and for evaluating potential strategies for prevention and cure.

ACKNOWLEDGMENTS

This work was supported by CRM RP award W81XWH-15-2-0024.



Elevated Expression of p21^{Cip1} in Blast Neurotrauma might Contribute to Blast-induced Tauopathy

Ying Wang, Yan Su, Yanling Wei, Donna M. Wilder, Irene D. Gist, Peethambaran Arun and Joseph B. Long

Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neurosciences, Walter Reed Army Institute of Research, Silver Spring, MD 20910

INTRODUCTION

The cyclin-dependent kinase inhibitor 1 (p21^{Cip1}) was initially characterized as a key inhibitor of the complexes of CDK2 and CDK1. Recent evidence suggests that an abnormal reactivation of the cell cycle may precede and cause the hyperphosphorylation and filament formation of tau protein in Alzheimer's disease and other tauopathies. Although significant increases in tau and phosphorylated tau (p-Tau) proteins have been widely described in multiple brain regions following blast-induced traumatic brain injury (bTBI) in rodents, the mechanism(s) underlying these changes is still undefined. To explore possible involvement of proteins involved in cell-cycle progression in these mechanisms, in the present study we investigated expression of these proteins in the rat cerebral cortex following exposure to the combination of blast with blunt brain injury. The effect of p21 regulation on Tau expression was further assessed in an in vitro experiment in which p21 CRISPR/Cas9 were transfected to the cultured primary neurons.

METHODS

Animal TBI model: Isoflurane anesthetized male Sprague Dawley rats, 350 g were positioned within the shock tube and exposed to a blast overpressure (19 psi static pressure) followed immediately by weight-drop (500g, 150 cm). Sham control animals were treated in the same fashion without exposure to blast.

Immunoblot Analyses: Cortex or cultured cells were homogenized in TPER or RIPA buffer containing protease and phosphatase inhibitors. Equal quantities of proteins were subjected to SDS-PAGE and western blot analysis using antibodies to Tau, p-Tau, p21 and actin.

Primary neuronal culture and treatment: Mouse cortices were dissected at embryonic day 18 and dissociated and cells were cultured in humidified 5% CO₂ incubator at 37°C serum-free Neurobasal medium containing B27 supplement.

Immunofluorescent staining: The coronal cerebral sections and cultured primary neurons were probed immunoreactive reactions with GFAP or Tau antibodies and incubated with Alexa Fluor 488-conjugated secondary antibody. Images were examined under a confocal microscope.

Statistical analysis: Data are presented as Mean ± SEM and the statistical analysis was carried out using Students *t* test.

RESULTS

Figure 1. Blast neurotrauma increased Tau and GFAP expression in cortex

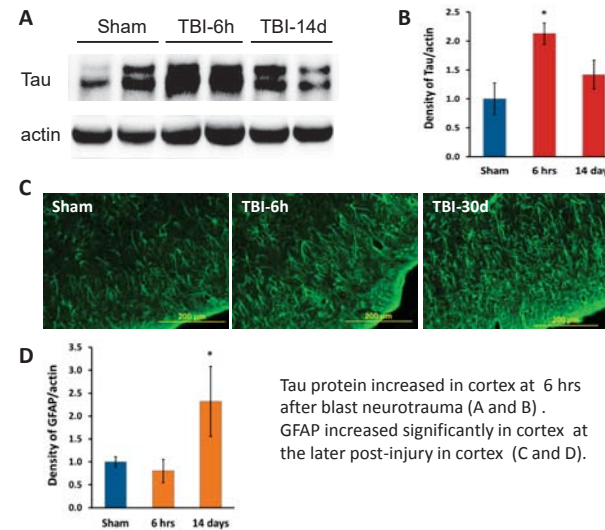


Figure 2. Blast neurotrauma induced p21^{Cip1} expression in cortex

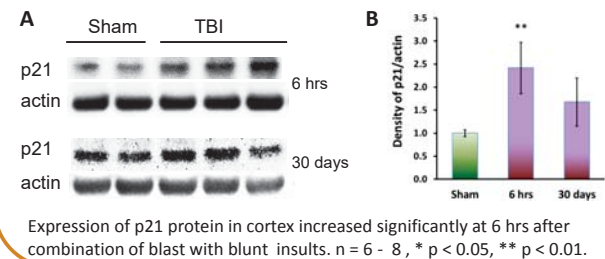


Figure 3. Up-regulation of Tau by overexpression of p21^{Cip1} in neuron

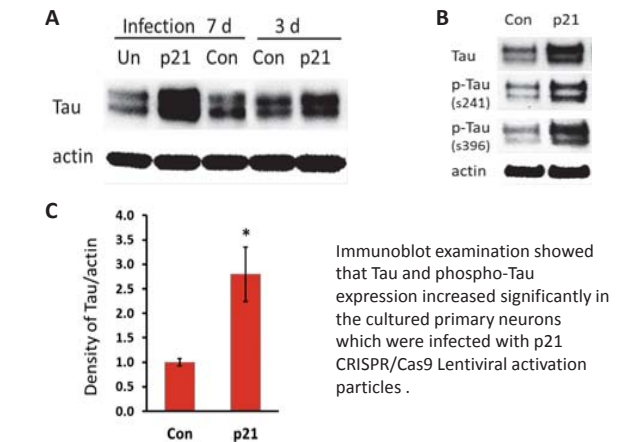
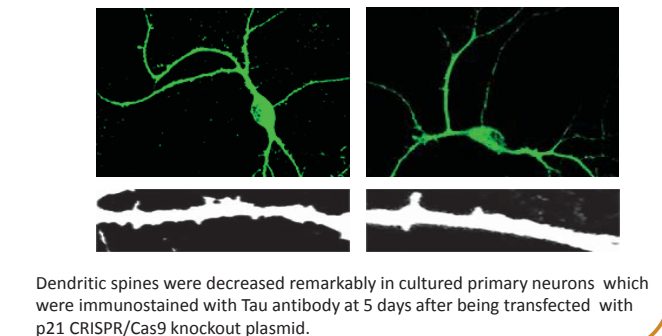


Figure 4. Decrease in Tau and dendritic spines by knockout p21^{Cip1}



SUMMARY

Expression of Tau and phospho-Tau can be up-regulated significantly by overexpression of p21^{Cip1}, and also can be down-regulated by knockout p21^{Cip1} in primary neuronal cultures. These combined results indicate that p21^{Cip1} may play significant roles in cytoskeletal dynamics and apoptosis in addition to cell cycle regulation. In particular, TBI-induced overexpression of p21^{Cip1} might contribute to tauopathy and might therefore serve as a potential therapeutic target for CTE mitigation.

ACKNOWLEDGEMENT

This study was supported by the CDMRP award W81XWH-11-2-0127, W81XWH-08-2-0018, and W81XWH-08-2-0017.

DISCLAIMER: Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted in an AAALACI accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 2011 edition.